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RIP1 has a role in CD40-mediated apoptosis in human follicular lymphoma cells

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ABSTRACT

CD40 is a cell surface receptor which belongs to tumor necrosis factor receptor (TNFR) family members. It transmits signals that regulate diverse cellular responses such as proliferation, differentiation, adhesion molecule expression and apoptosis. Unlike other TNFR family members (TRAIL-R, Fas-R and TNFR1), the CD40 cytoplasmic tail lacks death domain. However, CD40 is capable of inducing apoptosis in different types of cancer cells including lymphoma. The apoptotic effect of CD40 is linked to the involvement of Fas, TRAIL or receptor interacting protein 1 (RIP1) kinase. We have previously shown that CD40 activation has anti-apoptotic or apoptotic effect in follicular lymphoma (FL) cell lines. In this study, we investigated the mechanism by which CD40 mediates apoptosis in a follicular lymphoma cell line, HF4.9. We show here that CD40-induced apoptosis was dependent on caspase-8 activation because caspase-8 specific inhibitor, Z-IETD-FMK completely prevented apoptosis. Therefore, the involvement of TRAIL, Fas and RIP1 in caspase-8 activation was examined. The exogenous TRAIL-induced apoptosis was fully prevented by anti-TRAIL neutralizing antibody. However, the antibody had no effect on CD40-induced apoptosis indicating that CD40 did not induce the expression of endogenous TRAIL in HF4.9 cells. Moreover, the cells were not sensitive to Fas-mediated apoptosis. Interestingly, RIP1 specific inhibitor, necrostatin-1 decreased CD40-induced apoptosis, which showed that RIP1 has a role in caspase-8 activation. In conclusion, the survival or apoptotic effects of CD40-mediated signaling might be related to the differentiation stages of FL cells.

1. Introduction

CD40 is a transmembrane signaling molecule. It is expressed by a large variety of cell types such as B cells, dendritic cells, macrophages, endothelial cells and tumor cells. Whereas, CD40 ligand (CD40L) is mainly expressed on the activated $CD4^+$ T cells. The widespread expression of CD40 might indicate that it plays a broader role in human physiology and diseases pathogenesis (Kawabe et al., 2011; Elgueta et al., 2009a; Hassan et al., 2014).

CD40-CD40L interaction results in the activation of a variety of signaling cascades, which ultimately determine the diverse physiologic effects. For example, CD40 has a critical role in the development of adaptive immune responses. CD40 signaling rescues germinal center B cells from B cell receptor-mediated apoptosis and thereby promotes survival, selection and differentiation of B cells into memory and plasma cells (Elgueta et al., 2009b; Adem et al., 2015). Moreover, CD40 signaling has been linked to enhanced antigen presentation function and cytokine release (Companjen et al., 2002; Demangel et al., 2001). In contrast, a direct growth inhibitory effect of CD40 is observed in some neoplastic B-cells and different types of cancer cells (Tong and Stone, 2003; Funakoshi et al., 1994). The different mechanisms of CD40-induced elimination of cancer cells include induction of endogenous cytotoxic ligands and activation of anti-tumor immune responses (Eliopoulos et al., 2000; Dilloo et al., 1997). The phenotypic consequences of CD40 signaling, therefore, appear to be dependent on the cell types. However, the signaling pathways underlying the differential responses following CD40 activation are yet to be fully characterized.

Receptor-interacting protein 1 (RIP1) kinase is a multifunctional protein which contains N-terminal Ser/Thr kinase and a C-terminal death domain. It has context-specific roles in inflammation, cell survival and apoptosis (Knox et al., 2011). RIP1 is required for the pro-

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inflammatory and anti-apoptotic functions of tumor necrosis factor α receptor (TNFR). These RIP1 regulated cellular functions are mediated by nuclear factor κb (NF-κβ) and mitogen activated protein kinases (MAPKs) signaling (Festjens et al., 2007). RIP1 is also linked to CD40-induced apoptosis in carcinoma cells (Knox et al., 2011).

Apoptosis (programmed cell death) can be induced by a variety of stimuli including TRAIL and Fas. These death ligands activate their natural death receptors TRAIL-R and Fas-R respectively. The death receptors (DRs) have an intracellular death domain, which is essential for transduction of the apoptotic signal. The activation of DRs, therefore, lead to recruitment of the cytosolic adaptor protein, FADD and caspase-8, which form the death-inducing signaling complex (DISC). In the DISC, caspase-8 is activated and promotes apoptosis by activating the downstream effector caspases, caspase-3/7 (Cullen and Martin, 2009). Moreover, TNFR can also be involved in apoptosis. The activation of TNFR by TNFα can lead to the formation of complex IIa, which includes RIP1, caspase-8 and FADD. The interaction of these proteins within the complex results in the activation of caspase-8 and induction of apoptosis (Yuan and Kroemer, 2010). The cytoplasmic tail of CD40 lacks a death domain which mediates death signals by Fas and TRAIL receptors. Thus, the mechanisms of the CD40-inducced apoptosis pathway are not fully understood. Necrostatin-1 (Nec-1) is a specific inhibitor of RIP1 kinase and help to examine the requirement of this kinase in cell death (Ofengeim and Yuan, 2013).

MAPKs control a vast array of physiological and pathological processes such as cell growth, inflammation, apoptosis and autoimmunity. The three well-characterized MAPKs include p38MAPK, ERKs and JNK (Johnson and Lapadat, 2002). The p38 signaling pathway is linked to caspase-8 mediated apoptosis in human Burkitt lymphoma B-cells (Schrantz et al., 2001).

Follicular lymphoma (FL) cells represent neoplastic counterparts of germinal center B-cells (Kahl and Yang, 2016). Germinal centers (GCs) are specialized microanatomic structures of lymphoid organs which are formed when B-cells are activated by their specific antigen and receive costimulatory signals from helper T-cells. In GC, the activated B-cells undergo differentiation to centroblast, centrocyte, plasma cells/memory B-cells (De Silva and Klein, 2015; Oropallo and Cerutti, 2014). We have previously characterized FL cell lines by their immunophenotypic features and response to B-cell receptor stimulation and cytokines (Eray et al., 2003). Based on their unique features and responses, HF1A3 and HF4.9 cells can be used as an *in vitro* model for centrocyte and centroblast cells respectively. We have also previously shown that CD40 activation results in differential phenotypic responses in FL cell lines. In HF1A3 cells, CD40 protects cells from Fas- and TRAIL-mediated apoptosis (Eeva et al., 2007; Nuutinen et al., 2009). Whereas, in HF4.9 FL cells, CD40 activation leads to apoptosis (Nuutinen et al., 2009). In this study, we investigate the mechanism by which CD40 transduce death signaling molecule in follicular lymphoma cells, HF4.9. We report here that RIP1 has a role in CD40-mediated activation of caspase-8, which in turn leads to induction of apoptosis.

2. Materials and Methods

2.1. Cell line and culture conditions

The origin and characteristics of human follicular lymphoma cell line, HF4.9 has been previously described (Eray et al., 2003). The cells were cultured in RPMI 1640 medium (Lonza, Belgium) supplemented with 5% heat inactivated fetal bovine serum (GIBCO, Invitrogen, USA), 2 mM L-glutamine (Lonza), 106 U/ml streptomycin (Lonza), 106 U/ml penicillin (Lonza), 10 mM HEPS buffer (Lonza), 0.1 mM nonessential amino acids (Lonza), 1 mM Na-pyruvate (Lonza), 20 μM 2-mercaptoethanol (Fluka-Chemie, Buch, Switzerland) at 37 °C in a 5% CO_2 humidified atmosphere.

2.2. Cell treatments

HF4.9 cells were seeded on 12-well flat bottom polystyrene cell culture plates (Corning Inc, NY, USA) and treated with 50 μM caspase-8 specific inhibitor, Z-IETD-FMK (Calbiochem, San Diego, USA), 20 μM RIPK-1 inhibitor, necrostatin-1 (Calbiochem), 10 μM P38MAPK specific inhibitor, SB203580, (Cell Signaling technology, USA), 50 ng/ml His-tagged recombinant human soluble killer TRAIL™ (Enzo life sciences, Farmingdale, NY, USA), 0.25 μg/ml human TRAIL antibody (2E5) (Enzo life sciences), 10 ng/ml anti-CD95 (Fas) (Upstate Biotechnology, NY, USA). In order to rule out the immediate effects of serum on p38MAPK activation, the cells were incubated for 24 h and the treatment group was stimulated by 3.6 μg/ml anti-CD40 antibody for specific time points. In addition, anti-CD40 antibody was added to cell culture for the time indicated either alone or following 2 h pre-treatment with inhibitors. The anti-CD40 antibody was provided by Dr. Matti Kaartinen (Haartman institute, University of Helsinki, Finland).

2.3. Flow cytometric analysis of apoptotic cells

DNA fragmentation was determined by flow cytometric analysis after propidium iodide (PI) staining. Cells with fragmented DNA were considered as apoptotic. Fixation and staining of cells were performed according to a standard protocol. In brief, at the end of stimulation times samples containing one million cells were collected, resuspended in ice-cold PBS and fixed with ice-cold 70% v/v ethanol. After overnight incubation at + 4 °C, cells were centrifuged at 1500 RPM for 10 min, resuspended in PBS containing 150 μg/ml RNAase (Sigma, St. Louis USA) and incubated for 1 h at + 50 °C. PI (Molecular Probes, Sigma) was added to the final concentration of 8 μg/ml and incubation was further continued for 2 h at + 37 °C. FACSCanto II flow cytometer with FACSDiva version 6.1.2 software (Becton Dickinson, USA) was used for the analysis.

2.4. Preparation of whole cell lysates

At the end of stimulation times, two million cells were collected, washed in PBS and centrifuged at 1500 RPM for 5 min. The cell pellet was resuspended with lysis buffer containing 20 mM Tris–HCl (pH8.0), 2 mM EDTA, 3%NP-40, 100 mM NaCl, 50 mM NaF, 1 mM PMSF, 1 mM VO_4, 5 μg/ml aprotinin, and 5 μg/ml leupeptin. After 1 h of incubation on ice, samples were centrifuged at 13,000 RPM for 15 min, + 4 °C. The protein concentration of the cell lysates was measured, equalized with SDS-PAGE buffer (0.125 M Tris-Cl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, bromophenolblue) and boiled for 5 min.

2.5. Western blot analysis

Equal amount of protein from each sample was loaded and separated through 12% or 15% SDS-PAGE gel. Separated proteins were transferred to nitrocellulose membranes (GE Healthcare Life sciences, UK). Membranes were blocked with 1x TBS containing 3% BSA and 0.1% Tween-20 at room temperature. Then the membranes were incubated overnight at + 4 °C with the primary antibodies (anti-caspase-8, anti-caspase-3, phospho-P38MAPK, total p38MAPK and MAPKAPK-2). The membranes were washed three times with 1x TBS and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Zymed laboratories Inc, California, USA). Diluted in 3% BSA for 40 minutes at room temperature. The membranes were washed three times with 1x TBS. Finally, the membranes were developed for the appropriate time period that yields the desired results using enhanced chemiluminescence detection system (GE Healthcare Life sciences).

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5. The

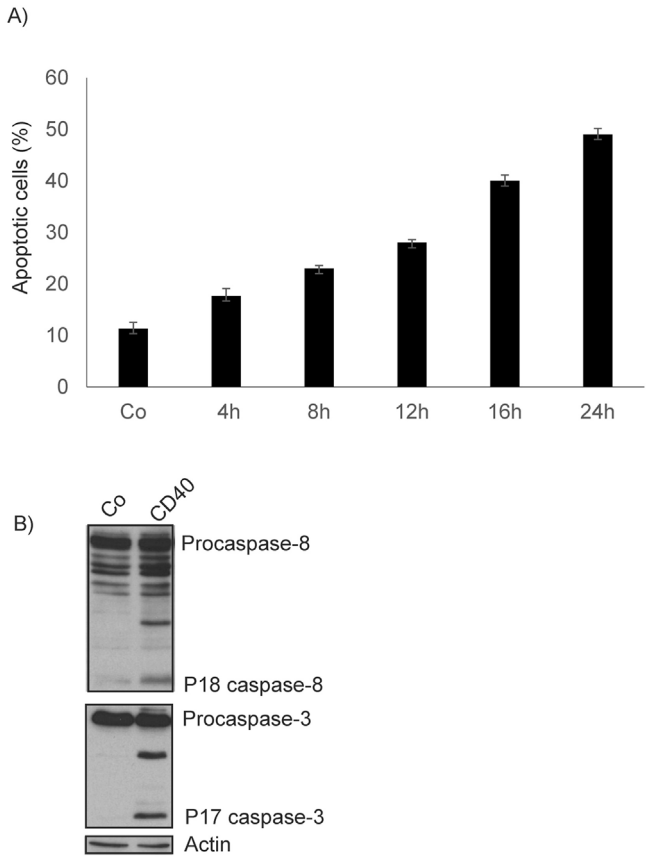

Fig. 1. Kinetics of CD40-induced apoptosis in HF4.9 cells. A) Cells were treated with anti-CD40 antibody for 4, 8, 12, 16 and 24 h. After incubation the cells were collected, fixed and stained with PI and analyzed by flow cytometry. Cells with fragmented DNA are considered as apoptotic. The data are presented as mean ± SEM from three independent experiments. B) Cells were treated with anti-CD40 antibody for 24 h. After stimulation the cells were collected and total cell lysates were prepared. The activation of caspase-8 and caspase-3 were detected by immunoblotting. Actin was used to ensure that there was equal amount of protein in each sample.

statistical difference between groups was determined with student's *t*-test. P value < 0.05 were considered statistically significant.

3. Results

3.1. CD40 stimulation triggers apoptosis through caspase-8 activation

CD40 stimulation in some lymphomas such as Burkitt lymphoma results in apoptosis through up-regulation of Bax, a pro-apoptotic Bcl-2 family protein (Szocinski et al., 2002). CD40 can also induce caspase-8 mediated apoptosis indirectly through death receptors (Eliopoulos et al., 2000). We have previously shown that CD40 activation in HF4.9 cells leads to apoptosis (Nuutinen et al., 2009), however, the mechanism was not investigated. Therefore, in this study we examined the possible pathways which could be involved in CD40-mediated apoptosis in HF4.9 cells. Cells were cultured in medium containing anti-CD40 antibody and collected at 4, 8, 12, 16 and 24 h. DNA fragmentation was measured by PI staining and cells with fragmented DNA were considered as apoptotic. The number of apoptotic cells started to increase after 4 h and continued to increase until 24 h (Fig. 1A). Next, we investigated whether apoptosis was mediated through activation of caspase-8 and caspase-3. Cells were incubated for 24 h with anti-CD40 antibody. At the end of stimulation time the cells were collected, proteins were isolated and immunoblotting was performed. Actin antibody was used to control that there was an equal amount of protein in each sample. Activated/cleaved caspase-8 and caspase-3 were detected (Fig. 1B). To determine whether caspase-8 was the only initiator of

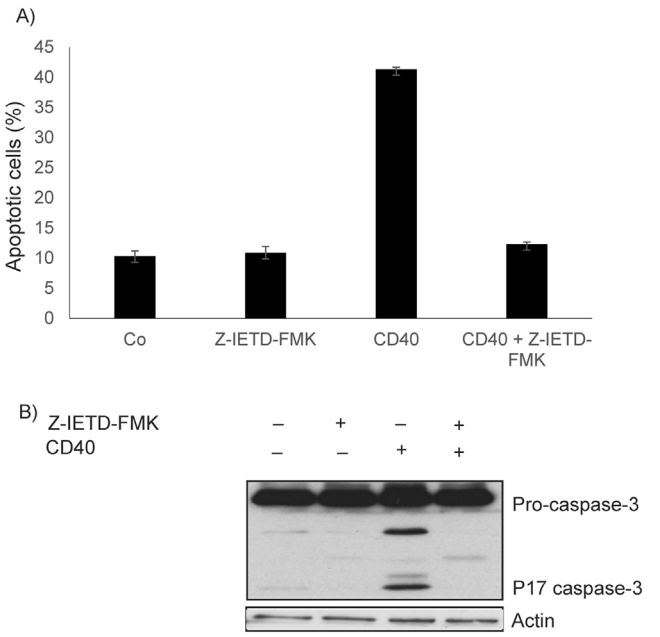

Fig. 2. CD40 mediates apoptosis by caspase-8 activation. A) Cells were treated with caspase-8 specific inhibitor, Z-IETD-FMK for 2 h prior to addition of anti-CD40 antibody for 16 h. After incubation the cells were collected for PI and western blot analysis. DNA fragmentation of cells was analyzed by flow cytometry after PI staining. The data are presented as mean ± SEM from three independent experiments. B) The activation of caspase-3 was detected by western blot. Actin was used to ensure that there was equal amount of protein in each sample.

caspase-3 activation, cells were treated with 50 μM Z-IETD-FMK (caspase-8 specific inhibitor) for 2 h prior to addition of anti-CD40 antibody for 16 h and collected for DNA fragmentation and immunoblotting analysis. Z-IETD-FMK completely blocked caspase-3 activation and apoptosis (Fig. 2A, B).

3.2. CD40-mediated apoptosis does not involve induction of death ligands

CD40 induces apoptosis in carcinoma cells through activation of cytotoxic ligands of the tumor necrosis factor superfamily (Eliopoulos et al., 2000). To examine this possibility in follicular lymphoma cells, we investigated the role of TRAIL and Fas in CD40-mediated apoptosis. TRAIL induced apoptosis in HF4.9 cells. However, cells were not sensitive to Fas (Fig. 3C). To determine the role of endogenous TRAIL in CD40-mediated apoptosis, we used anti-TRAIL antibody (2E5). Cells were incubated with TRAIL, anti-CD40 antibody, and/or 2E5 antibody for 16 h. The 2E5 antibody was added 2 h prior to addition of TRAIL or anti-CD40 antibody. After incubations cells were collected and DNA fragmentation was measured by PI-staining. The 2E5 antibody completely prevented TRAIL-induced apoptosis. Whereas, the antibody had no effect on CD40-induced apoptosis confirming that endogenous TRAIL was not induced.

3.3. CD40-induced apoptosis requires RIP1

To identify the pathway leading to caspase-8 activation upon CD40 stimulation, we examined the role of RIP1 kinase which has been linked to CD40-mediated apoptosis (Knox et al., 2011). Cells were incubated with RIP1 specific inhibitor (Nec-1) and/or anti-CD40 antibody for 16 h. After incubation cells were collected for PI-staining and immunoblotting. Nec-1 partially inhibited apoptosis, as the proportion of apoptotic cells decreased from 40% to 25%, ($p < 0.001$). The decrease in apoptosis also correlated with the level of active caspase-3 (Fig. 3A, B)

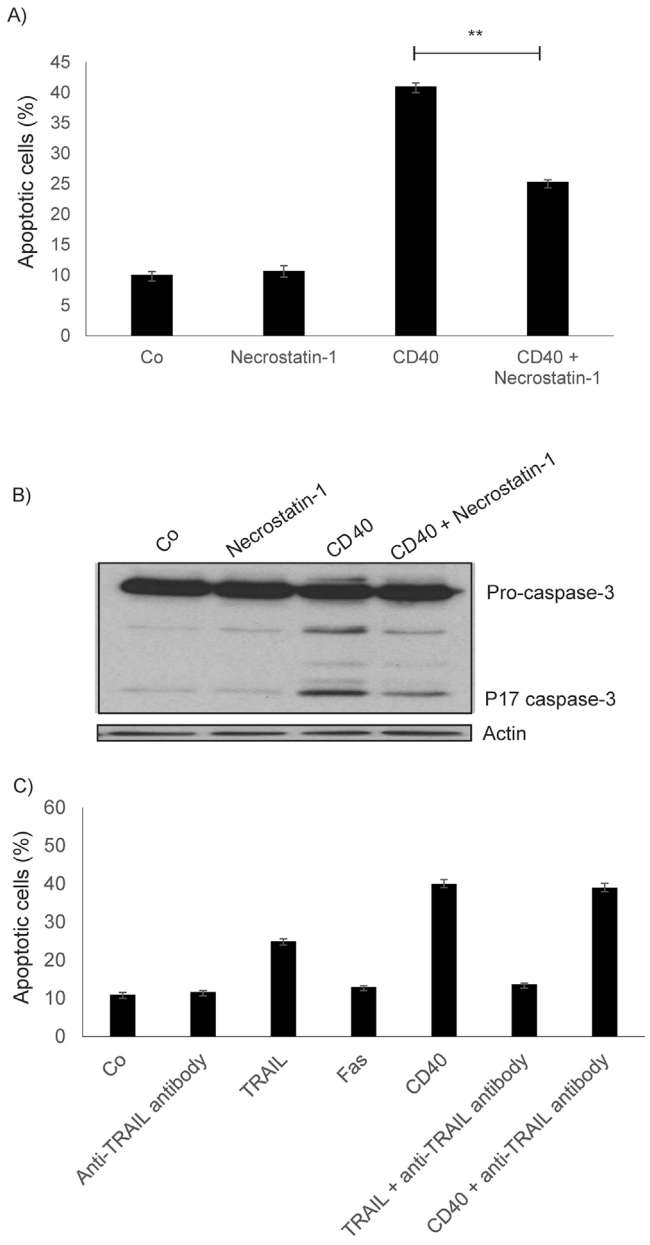

Fig. 3. RIP1 has a role in CD40-mediated apoptosis in HF4.9 cells. A) Cells were treated with RIP1 specific inhibitor, Necrostatin-1 for 2 h prior to addition of anti-CD40 antibody for 16 h. After incubation the cells were collected for PI and western blot analysis. Cells with fragmented DNA were analyzed by flow cytometry. The data are presented as mean ± SEM from three independent experiments. The statistical significance of difference was determined using student's *t*-test; $^{**}p < 0.001$. B) The activation of caspase-3 was detected by western blot. To control the equal amount of protein was loaded on the gel, antibody against actin was used. C) Cells were treated with TRAIL, anti-CD40 antibody and Fas. Cells were also treated with anti-TRAIL antibody for 2 h prior to addition of TRAIL. After incubation time the cells were collected for PI analysis. The data are presented as mean ± SEM from three independent experiments.

3.4. CD40-induced caspase-8 activation is not dependent on p38 MAPK

P38 MAPK activation by TGFβ involves FADD-independent activation of caspase-8 (Schrantz et al., 2001). We therefore examined the activity of p38 MAPK and its effect on apoptosis. Cells were incubated with anti-CD40 antibody for 1, 4, 8, 16 and 24 h. After incubations cells were collected, proteins were isolated and immunoblotting was performed using phospho-p38 MAPK and total p38MAPK antibodies. Interstingly, CD40 stimulation led to phosphorylation of p38MAPK, which was detected from the earliest time points until end of

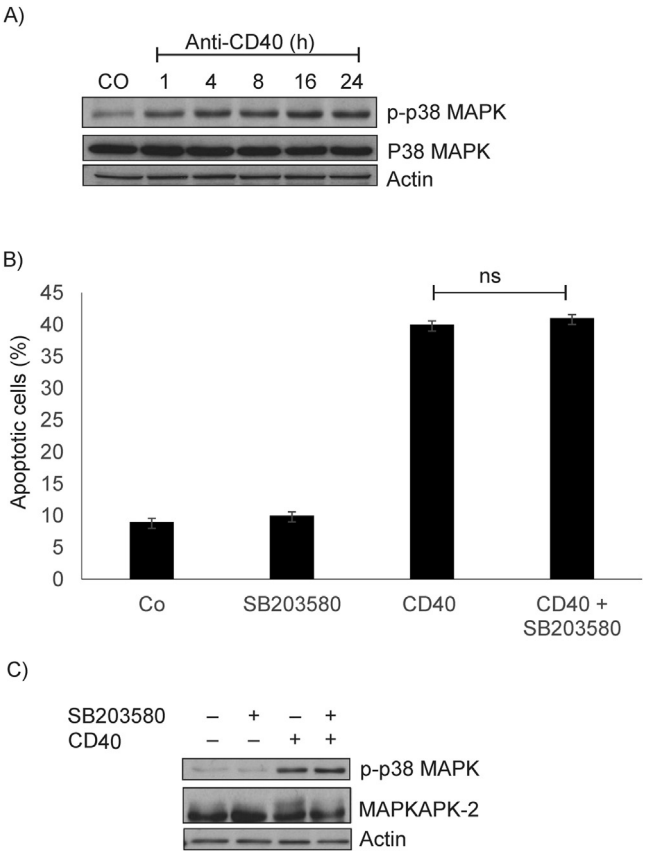

Fig. 4. p38MAPK has no involvement in CD40-induced caspase-8 activation. A) Cells were treated with anti-CD40 antibody for 1, 4, 8, 16, and 24 h. After incubation time the cells were collected for western blot analysis. The level of phospho-p38MAPK and total p38MAPK were detected by western blot. B) Cells were treated with p38MAPK specific inhibitor, SB203580 for 2 h prior to addition of anti-CD40 antibody for 16 h. After incubation the cells were collected for PI and western blot analysis. The data are presented as mean ± SEM from three independent experiments. The statistical significance of difference was determined using student *t*-test. C) The levels of phospho-p38MAPK, MAPKAPK-2 and actin were detected by western blot.

stimulation (Fig. 4A). Therefore, we investigated the role of phospho-p38MAPK in CD40-mediated apoptosis by using p38MAPK specific inhibitor, SB203580. Cells were treated with 10 μM SB203580 for 2 h prior to addition of anti-CD40 antibody for 16 h. Cells were collected for DNA fragmentation and immunoblotting analysis. Interstingly, CD40-induced phosphorylation of MAPKAPK-2 (the downstream target of p38MAPK) was detected as a phosphorylation specific MAPKAPK-2 band shift on SDS-PAGE. Moreover, the phosphorylation was inhibited by SB203580 but SB203580 did not prevent CD40-induced apoptosis (Fig. 4B, C).

4. Discussion

CD40 is expressed by hematopoietic and non-hematopoietic cells (Elgueta et al., 2009a). Although, it has important pro-survival functions in the development of humoral immunity, CD40L-CD40 interaction in some cell types leads to the induction of apoptosis. In the present study, we examined the apoptotic pathway involved in CD40-mediated apoptosis in human follicular lymphoma (FL) cells. As CD40-induced apoptosis in HF4.9 cells is fully prevented by the inhibition of caspase-8 activation, we focused on cellular death inducing proteins which have a key role in caspase-8 regulation. In this regard, the role of RIP1, TRAIL, Fas and p38MAPK was examined. We report here that TRAIL, Fas and p38MAPK have no role in CD40-induced apoptosis, whereas RIP1 was involved in apoptosis because RIP1 inhibition by Nec-1 partially prevented the apoptosis. The inability of Nec-1 to completely abolish

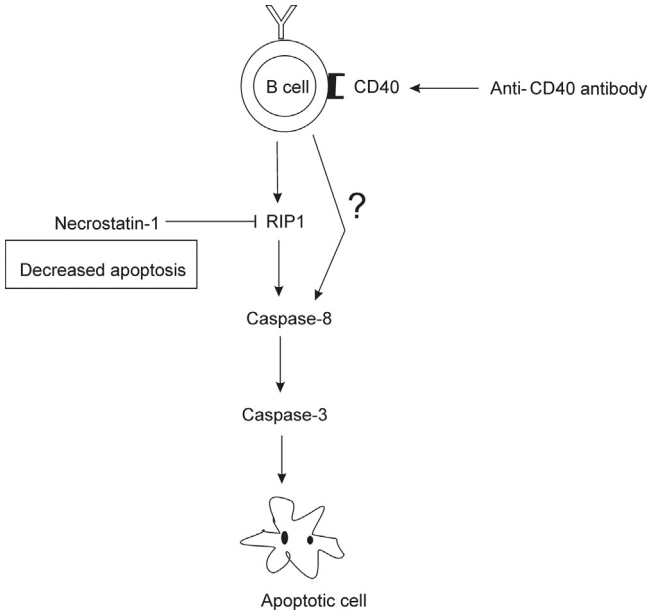

Fig. 5. Model of CD40-induced apoptosis in HF4.9 cells. Stimulation of CD40 activates caspase-8 and caspase-3 which leads to apoptosis is in HF4.9 cells. Inhibition of RIP1 activity by Necrostain-1 partially prevents CD40-induced apoptosis. Interstingly, Fas, TRAIL and p38MAPK has no role in CD40-mediated caspase-8 activation. These findings indicate that other proteins/kinases are involved in the activation of caspase-8.

CD40-mediated apoptosis indicates that caspase-8 is activated by additional death signals. Consistent with our findings, the importance of RIP1 in CD40-mediated apoptosis in carcinoma cells has been demonstrated (Knox et al., 2011). Moreover, CD40 ligation is found to induce endogenous cytotoxic ligands (Fas and/or TRAIL) in carcinoma cells and hepatocyte (Eliopoulos et al., 2000; Afford et al., 1999). Therefore, the ability of CD40 to induce these death ligands seems to be cell type specific. Altogether, these data demonstrate that there are multiple proteins/kinases which regulate CD40-mediated caspase-8 activation.

It is well known that RIP1 is crucially important in development and maintenance of B-cells (Zhang et al., 2011). However, RIP1 has also contradicting cellular functions such as cell survival or apoptosis. Our data strengthen the fact that the different cellular responses following CD40 stimulation in GC-derived B-cells could be determined by maturational stages of the cells. GCs are microanatomic structures where B-cells proliferate, mutate their receptor genes, differentiate, and switch isotype of their antibodies. The GC structure is comprised of a dark zone (DZ) and a light zone (LZ). DZ contains highly proliferating B-cells (Centroblasts). These cells undergo somatic hypermutation (SHM). SHM increases BCR diversity and thereby increases chances of producing B-cells with high affinity to antigens. However, SHM has also a downside, because it leads to the production of B-cells which are capable of recognizing self-antigens. In the LZ, therefore, B-cells (centrocytes) are subjected to selection. T helper cells-derived co- stimulatory signals such as CD40 are delivered to non-self-reactive B-cells to rescue them from elimination (De Silva and Klein, 2015; Oropallo and Cerutti, 2014). We have previously shown that CD40 rescues HF1A3 cells from B-cell receptor mediated apoptosis (Adem et al., 2015; Eeva et al., 2003). Based on our findings, the different outcome of CD40 signaling in HF1A3 (representing centrocyte) and HF4.9 (representing centroblast) (Eray et al., 2003) cells seems to depend on their developmental stage. Therefore, we speculate that CD40-mediated signaling might induce apoptosis in cells which undergo SHM, thereby preventing the production of self-reactive B-cells.

In summary we have shown that anti-CD40 antibody induces RIP1 dependent apoptosis in human follicular lymphoma cells, HF.49. The pro-apoptotic function of RIP1 is independent of the involvement of cytotoxic ligands. These findings may also provide insights into the role of RIP1 in differential stages of germinal center B-cells. In addition to RIP1, CD40-induced signaling pathways which regulate caspase-8 activation will be an interesting area for future studies (Fig. 5).

Conflicts of interest statement

The authors declare no financial or commercial conflict of interest.

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